Versatile microbial surface-display for environmental remediation and biofuels production

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Abstract

Surface display is a powerful technique that utilizes natural microbial functional components to express proteins or peptides on the cell exterior. Since the reporting of the first surface-display system in the mid-1980s, a variety of new systems have been reported for yeast, Gram-positive and Gram-negative bacteria. Non-conventional display methods are emerging, eliminating the generation of genetically modified microorganisms. Cells with surface display are used as biocatalysts, biosorbents and biostimulants. Microbial cell-surface display has proven to be extremely important for numerous applications ranging from combinatorial library screening and protein engineering to bioremediation and biofuels production.

Introduction

An increased understanding of microbial genomes and proteomes in recent years along with advances in recombinant technology significantly improved our ability to manipulate microorganisms for biotechnological applications. In particular, the ability to express heterologous proteins on the cell surface has become the foundation for a wide range of important medical and environmental applications. The first use of surface display was in the development of vaccines using a filamentous phage coat protein [1] and the *Escherichia coli* membrane protein, LamB [2], to express antigens on the cell surface. The natural functions of surface proteins are advantageous for anchoring 'passenger' proteins on cell membranes. Soon after, it was realized that display of functional proteins on the cell surface provided a promising technique for the improvement of conventional biocatalysts. As a result there was a corresponding increase in the published literature in the 1990s on the development of different host cells, carrier proteins and applications. The main advantages of surface-expressed over intracellularly-expressed proteins include the elimination of the target compound crossing the membrane barrier, thus removing the rate-limiting step and increasing the overall kinetics; stabilization of enzymes and proteins through attachment to cells to increase yield and simplification of the protein purification step by centrifugation or settling of cells. Current challenges in surface display research include expressing large multimeric proteins greater than 60 kDa, displaying multiple proteins and alleviating the spread of genetically modified organisms in live-vaccine and field applications.

Microbial cell-surface display has a myriad of applications such as industrial catalysts, sorbents, sensors, vaccine delivery vehicles and screening platforms (Figure 1).

Remediation of pollutants and biofuels production are two active research areas using surface-displayed biocatalysts and biosorbents. Contamination of soil and water threatens the well-being of humans and natural flora and fauna. Clean-up of polluted sites is a priority for governments around the world. Remediation is a multi-million dollar industry and cost effective methods are constantly being explored. Most of the pollutants can be categorized into inorganic compounds and recalcitrant organic compounds. Microbes with surface expressed proteins are used to accelerate adsorption of heavy metal contaminants and degradation of organic compounds.

Additionally, viable alternative fuel sources are needed to reduce continued geopolitical tension over fossil fuel supplies. Biocatalysts with surface-displayed enzymes capable of converting plant biomass substrates into ethanol and butanol hold promising potential. Alcohol-based fuels would also lessen petroleum by-product contamination of the environment.

There are a number of excellent recent reviews describing different types of microbial cell surface display systems for Gram-positive bacteria, Gram-negative bacteria, and yeast, as well as current applications [3-8]. In this review, we focus on the application of this technology for bioremediation and biofuels production (Table 1), and highlight new developments in the field.

Surface-display for inorganic pollutant remediation

Inorganic pollutants such as heavy metals and radionuclides are recalcitrant and do not disintegrate. Therefore, sequestration and/or immobilization are ideal remediation

strategies. Surface expression of metal binding proteins or peptides enables rapid binding of heavy metals within minutes, and improves metal resistance of growing cultures.

An example of binding of heavy metals involves the *de novo* construction of a synthetic phytochelatin (EC), a repetitive metal-binding motif consisting of $(Glu\text{-}Cys)_nGly$ [9]. These peptides emulate the structure of phytochelatin, a naturally occurring metal-chelating peptide that plays a major role in plant metal detoxification. *E. coli* with cell surface-expressed EC exhibit higher cadmium (Cd^{2+}) and mercury ion (Hg^{2+}) accumulation than cells with intracellularly-expressed EC by 12-fold [9] and 20-fold [10], respectively.

In addition to *de novo* designed peptides, metalloregulatory proteins, such as MerR, are another group of useful metal-binding proteins with high affinity and specificity [11, 12]. Surface-expressed MerR using the ice nucleation protein (INP) anchor binds 6-fold more Hg^{2+} than the wild-type *E. coli* cells [13]. Mercury sorption via MerR is highly specific, with no observable decline in the presence of 100-fold excess Cd^{2+} and Zn^{2+}. In another study, a metal-binding domain (MBD), consisting of MerR dimers, was surface-displayed using the lipoprotein–outer membrane protein anchor (Lpp–OmpA). *E. coli* cells displaying MBD bound 6-fold more Hg^{2+}, and were protected from mercury toxicity [14].

Another advantage of using cell surface display technology is the rapid high-throughput screening of libraries. *E. coli* expressing a random dodecapeptide on the cell surface anchored by the flagellar protein, FliC, was used to screen a peptide library for nickel biosorption [15]. Peptides were screened by two rounds of selection using Ni-NTA HisSorb Strips to separate the nickel-bound from the non-nickel-bound peptides.

This high-affinity peptide selection strategy can be applied to sorption of other heavy metals of interest.

Biosorbent yeast cells have GRAS (generally regarded as safe) status and are capable of displaying large proteins. A yeast metallothionein (YMT) was displayed on the yeast cell surface using α-agglutinin [16]. Metallothioneins are cystein-rich proteins with high affinity towards metals. A comparison of different numbers of YMT tandem repeats indicated that a higher number of YMT expressed corresponded to higher amount of cadmium bound on the yeast cells, and increased tolerance to cadmium toxicity. The flexibility in displaying multiple YMT binding domains on yeasts creates high capacity biosorbents useful for large-scale heavy metal remediation.

Surface-display for organic pollutant remediation

Organic compounds degrade through microbial-mediated or chemical processes. However, certain anthropogenic chemicals that do not exist in nature are recalcitrant because no naturally occurring enzymes break down these compounds. Biocatalysts based on surface-display enzymes could improve remediation efficiency of these deleterious xenobiotics by eliminating the transport barrier across the cell membrane. Surface displayed microbes have been used to remediate organic pollutants such as organophosphates. *E. coli* cells expressing organophosphorus hydrolase (OPH) on the cell surface degraded parathion and paraoxon 7-fold faster than whole cells expressing OPH intracellularly [17]. The resulting live biocatalysts are also considerably more stable and robust than purified OPH, retaining 100% activity over a period of one month [17]. Recently, Takayama and co-authors [18] demonstrated OPH expression on the

yeast cell surface using a fusion of the α-agglutinin secretion signal and the glycosylphosphatidylinositol (GPI) anchor attachment signal. Yeast cells displaying OPH exhibited 10-fold higher activity than the surface expressed OPH in *E. coli* [19], suggesting that yeast may be a more suitable host for future engineering applications.

In addition to bacterial enzymes, a mosquito insecticide resistance-associated carboxylesterase (CaE B1), was anchored on the *E. coli* cell surface using the INP anchor. Cells with surface-displayed CaE B1 rapidly degraded 90% of malathion achieved within four hours [20]. This is substantially faster than intracellularly-expressed OPH which has limited activity towards malathion.

Directed evolution has been used to improve OPH activity towards poorly degraded substrates such as methyl parathion and chlorpyrifos. In two rounds of DNA shuffling and whole-cell screening using surface-displayed OPH variants, more than 50-fold and 750-fold improvements in activity were observed for methyl parathion and chlorpyrifos, respectively [21].

The ultimate goal of creating bioremediating biocatalysts is to stimulate the break down of organic xenobiotics in the field. Microorganism populations that are sustainable in nutrient-limiting conditions, such as in soils and groundwater, are necessary for effective attenuation. *Pseudomonas putida* and *Moraxella* sp., commonly found in the environment, have been engineered to display OPH on the cell surface[22-24], and shown to exhibit 70-fold higher activity than that observed in *E. coli* [25].

Another approach to sustain bacterial populations in the soil is through exploitation of the synergistic plant–microbe relationship in the plant root. This rhizoremediation strategy has been demonstrated using a wheat rhizosphere system for

the detoxification of soil trichloroethylene (TCE) [26]. The toluene *o*-monooxygenase gene was introduced into *Pseudomonas fluorescens* 2-79, a bacterium that colonizes the wheat root, enabling the establishment of a bacterium–plant symbiosis. Treatment of TCE-contaminated surface soil was demonstrated, with more than 63% of the initial TCE removed within four days.

Over 40% of all Superfund sites in the U.S. are co-contaminated with organic pollutants and heavy metals. Strategies to address the mixed-waste situation require the use of rhizobacteria that will survive and thrive in soils heavily polluted with heavy metals. Expression of metal-binding and TCE-degrading proteins in bacterial cells confers metal-resistance and allows retention of TCE degradation functionality in the presence of high metal concentrations. Engineered *Rhizobium sp.* with surface displayed EC was shown to be resistant to elevated cadmium concentration and retained its TCE degrading activity [27]. The TCE degradation rate for cells without EC expression was reduced by 60% in the presence of cadmium. With the expression of EC, TCE degradation rate was restored to the same level as no cadmium addition, and cadmium accumulation was also observed. In another study, sunflower roots were inoculated with engineered rhizobacterium expressing EC resulting in a marked decrease in cadmium phytotoxicity and a 40% increase in cadmium accumulation in the root rhizoplane [28]. Inoculation of these engineered rhizobacteria into plant roots could provide a valuable solution for mixed-waste remediation by reducing effects of heavy metal toxicity.

Another potential mix-waste remediation strategy involves cyclodextrin glycosyl transferase (CGTase), an enzyme ranging from 60 to 110 kDa. The CGTase from *Bacillus circulans* was anchored onto the yeast cell surface through the α-agglutinin core

unit (AGA1) linked to a small binding subunit (AGA2) [29]. The functions of CGTase include hydrolysis of starch, cyclicalization of non-reducing dextrins, coupling and disproportionation of oligosaccharides and modification of the length of non-cyclic dextrins [30]. Yeast cells with surface-expressed CGTase enabled yeast to utilize starch as a carbon source to produce 24 mg/ml of cyclodextrins. The cylindrical shape of cyclodextrins, which is hydrophobic inside and hydrophilic outside, allow it to form complexes with hydrophobic compounds. The ability to immobilize hydrophobic molecules within the ring structure of cyclodextrins makes the molecule suitable for a range of applications including simultaneous remediation of organic compounds and heavy metals [31], as well as starch hydrolysis for biofuels production [30].

Surface-display for biofuels production

Lignocellulosic materials are the most abundant form of stored renewable energy. The production of ethanol from plant biomass is a promising sustainable energy source [32]. The current process of biofuels generation from plant materials is a multi-step process involving breakdown of biomass, separation of lignin, hemicellulose and cellulose (pretreatment), hydrolysis of lignocellulose to monosaccharides (saccharification) and conversion of sugars to alcohol (fermentation). Biofuels production is in most need of improvement in the areas of pretreatment of biomass and hydrolysis of cellulosic materials [33]. Next to feedstock production, thermochemical biomass pretreatment has the highest energy cost in the process [34]. The improvement of starch hydrolysis by biocatalysts is crucial for reducing energy input at this stage. Lignocellulose consists of cellulose and hemicellulose polymers bound by lignin.

Specific limitations include difficulty in the breakdown of lignin to release cellulose and hemicellulose and the low efficiency in fermentation of pentose sugars such as xylose, which is a major constituent of the monosaccharides produced. Current research is moving towards consolidating cellulase production, cellulose hydrolysis and fermentation into one step (consolidated bioprocessing) using a single organism. It has been demonstrated that cellulose–enzyme–microbe complexes yield much higher rates of cellulose hydrolysis than using purified enzymes [35].

A viable solution to address the limitations is through surface display of various cellulases, combined with fermentation genes in a robust organism such as *Saccharomyces cerevisiae*. A recent paper comparing cellobiose usage by surface expressed versus secreted β-glucosidase demonstrated that expression on the yeast cell surface stabilized and increased specific activity of the enzyme [36]. Fujita et al., [37] reported co-display of endoglucanase II and β-glucosidase on the surface of *S. cerevisiae* MT8-1. The strain was able to grow on β-glucan as the sole carbon source and produced 16.5 g/l of ethanol without pretreatment, whereas the strains without the co-displayed enzymes did not grow. The same yeast strain, *S. cerevisiae* MT8-1, was also engineered to express three xylose fermenting enzymes intracellularly and β-glucosidase extracellularly [38]. Ethanol production from xylose, cellobiose, sugar mixture and lignocellulosic hydrolysate were compared. The recombinant strain produced slightly higher amounts of ethanol (30 g/l) than the wild type strain (22 g/l) when grown on lignocellulosic hydrolysate as the sole carbon source. However, the additional display of β-glucosidase on the surface did not improve ethanol production compared to the strain

with only the intracellularly-expressed xylose fermenting genes. This may be due to the diminished activity of the surface expressed enzyme.

Amylases facilitate break down of starch carbohydrates to maltotriose, maltose, glucose and dextrin. Glucoamylase from *Rhyzopus oryzae* was expressed on the yeast surface and grown solely on starch in batch, repeated-batch and fed-batch fermentation cultures. The fed-batch yielded the highest amount of ethanol (50 g/l) [39]. In another study, co-display of glucoamylase and amylase from *Bacillus stearothermophilus* produced higher amounts of ethanol than surface display of glucoamylase and secreted amylase [40, 41].

A yeast-based biocatalyst displaying *Trichoderma reesei* xylanase II (XYNII) on the cell-surface provided the cells with the ability to degrade xylan [42]. Xylanase breaks down hemicellulose into xylose. Subsequent research from the same group co-displayed XYNII and β-xylosidase (XylA) on the yeast cell surface and intracellularly expressed three xylose fermentation genes (XYL1, XYL2, XKS1) [43]. The yeast strain was capable of producing 7.1 g/l of ethanol from birchwood xylan and the yield was further improved to 30 g/l ethanol with chemically pretreated woodchip [38].

In the studies discussed above, the enzymes were displayed on the surface by using the C-terminal half of the α-agglutinin. Recently, a different α-amylase, from *Streptococcus bovis* 148, which has the potential to hydrolyze starch at a higher rate was co-displayed on the yeast cell surface using the flocculation functional domain (Flo1p). The engineered strain of starch hydrolyzing yeast, co-displaying glucoamylase and α-amylase, was able to use raw corn starch as a carbon source to produce 61.8 g/l of ethanol in 72 hours [44].

The main advantage of surface expressed lignocellulolytic enzymes is clearly the extracellular digestion of complex carbohydrates eliminating the need for transport of large polymers across the cell membrane. A strain of *Zymobacter palmae* was engineered to express xylose catabolic enzymes intracellularly, and produce approximately 45 g/l of ethanol from pure xylose [45]. The ability of the strain to utilize corn starch or more complex carbohydrates as a carbon source was not explored. Therefore, the comparison of *Z. palmae* with previous described surface display biocatalysts cannot be made. The potential exists for significant improvement of *Z. palmae* ethanol production from raw biomass with surface expression of lignocellulolytic enzymes such as amylases or xylanses. Current research on biofuels production with surface displayed biocatalysts is moving towards simultaneous saccharification and fermentation, and one step closer towards achieving consolidated bioprocessing [35].

Recent developments in cell surface display technology

Since the reporting of the first microbial membrane protein manipulation to display a non-native protein, the database for new protein anchors continues to grow. Despite familiarity with well-studied systems, the discovery for novel display carriers is necessary to accomplish more complex functions. One approach to aide in the search for new carriers is through understanding the functions of native surface proteins involved in pathogenesis, nutrient acquisition, motility, mating, cell-wall maintenance and protection (Figure 2). Many recently developed microbial anchor proteins that are useful for environmental remediation and biofuel production will be discussed.

New cell-surface anchors

Autotransporters are proteins secreted through the periplasm and anchored to the outer membrane. The *Neisseria gonorrhoeae* IgA protease is one of the earliest anchor proteins used for microbial surface display [5, 46]. More recent autotransporter anchors include the *E. coli* AIDA-I transporter protein [47] and the *Pseudomonas aeruginosa* EstA outer membrane esterase [48, 49]. Functional display of OPH-GFP has been demonstrated recently using the AIDA-1 transporter [50]. The resulting strain is useful for simultaneous pesticide degradation and on-site monitoring.

Flagella are cell surface appendages that serve the purpose of providing motility. The *E. coli* flagellar filaments, such as the FliCD proteins, have been exploited for expression of foreign proteins and peptides [15, 51]. The five FliD molecules that make up the capping structure at the end of the flagellum were used to simultaneously display fibronectin, the collagen-binding YadA, and the surface-layer (S-layer) protein, SlpA, of *Lactobacillus brevis* [51]. The display of multiple degradative enzymes is advantageous for specifically targeting sequential pollutant degradation or cellulose hydrolysis.

S-layer proteins comprise two-dimensional arrays with a crystalline appearance. S-layer proteins have been detected across all phylogentic groupings in bacteria and archaea, and might function as molecular sieves, a protective shield and/or virulence factors [4, 52]. The S-layer protein of *Caulobacter crescentus*, RsaA, was used to express protein G on the surface of *C. crescentus* cells [53]. This will provide a low-cost method for antibody binding, with applications in the development of immunoassays.

A recent study reported the expression of a *Burkholderia gladioli* carboxylesterase, EstA, by in-frame fusion to endogenous yeast proteins.

Kre1p/EstA/Cwp2p and Kre1p/EstA/Flo1p fusions expressed on the yeast surface were compared [54]. Kre1p is the toxin membrane receptor required for lethal ion channel formation [55], Cwp2p is essential for normal cell wall formation [56], and Flo1p is responsible for flocculation [57]. The Kre1p/EstA/Cwp2p fusion system expresses 60-fold higher EstA activity than *E. coli* expressing esterase A using the bacterial autotransporter system. This high-level expression system can be advantageous for improving the rate of catalysis for many practical applications.

In addition to using naturally existing surface proteins, rational design is also used to generate a novel surface display scaffold. The *E. coli* outer membrane protein OmpX was circularly permutated (CPX) to display both N and C termini on the cell exterior [58]. The purpose is to tether peptides on the cell-surface via a flexible linker fused to a single terminus to mimic peptide solution behavior in the absence of the scaffold. Passenger proteins were displayed at either the N or the C terminus, and the isolated peptides using the CPX scaffold had higher affinity for the target than peptides selected with insertional fusion libraries. *E. coli* cells simultaneously expressing GFP intracellularly and streptavidin-binding peptide extracellularly with CPX were immobilized onto a microfabricated electrode array using positive dielectrophoresis (DEP), thus allowing each sensor element to be measured electrically for multiple ligand display technology [59]. This system will be used for selecting high-affinity binders for a wide range of heavy metals.

There are also non-conventional techniques for surface-display instead of through the transcriptional machinery. Tanaka et al. [60] employed an antimicrobial peptide, temporin, that spontaneously imbeds itself into cell membranes to serve as a membrane

anchor for display of streptavidin on bacterial magnetic particles (BMP). The antimicrobial mechanism of temporin involves membrane permeabilization and cell lysis by the interaction of peptides with the cytoplasmic membrane. Unfortunately, expression of temporin fused to surface protein on live cells will ultimately lead to cell damage or death. Moreover, the ability of temporin to integrate into the membrane after fusing to a passenger peptide remains to be seen.

Another cell wall precursor UDP-MurNAc pentapeptide coupled with oligomannose was applied successfully to cell surface display [61]. *Lactobacillus plantarum* was grown in medium containing the bacterial cell-wall precursor and surface display of mannose was confirmed through adhesion to the ConA (mannose-binding-protein)-immobilized surface. Both of these non-transcription-based anchor methods eliminate the use of genetically modified organisms for surface display, and could alleviate the concern associated with using recombinant cells for *in situ* remediation. It is possible that temporin-BMP or the *L. plantarum* displaying metal binding peptides can be developed into environmentally safe biosorbents for heavy metal removal.

New hosts for protein anchor and safety issues of GMO use

In addition to Gram-positive bacteria, Gram-negative bacteria, and yeast, alternative non-self propagating hosts such as *Bacillus subtilis* spores, non-living *Lactococcus lactis* and bacterial magnetic particles (BMP) have been exploited in cell surface display.

Spores can survive indefinitely in a metabolically inactive state, stay intact for millions of years and resist temperatures as high as 90ºC [62]. *B. subtilis* spores

displaying the tetanus toxin fragment C using the spore outer coat protein CotB were used as live vaccines [62, 63], as well as for screening of tetrameric streptavidin [64]. Another approach is the use of chemically pretreated and boiled *L. lactis* as a matrix to bind heterologous proteins externally [65]. The protein peptidoglycan hydrolase, AcmA, was demonstrated to display α-amylase and β-lactamase, as well as epitopes of the *Plasmodium berghei malaria* circumsporozoite protein antigen. Nasally immunized mice with the non-living, non-recombinant vaccine showed higher levels of immunoglobulin G (IgG)-specific serum antibodies after the second dosing than the subcutaneously immunized mice.

BMP from *Magnetospirillum magneticum* AMB-1 [66, 67] is another host cell with the unique advantages of stability with magnetic separation capability. New BMP membrane proteins and promoters are being identified based on whole genome sequence and proteome analysis [68, 69]. Integral BMP membrane proteins (e.g. Mms16, Mms13) were used recently for stable display of large proteins such as immunoglobulin G-binding domain of protein A (ZZ) [67], G protein-coupled receptors (GPCR) and the D1 dopamine receptor [66]. Mms13-anchored-ZZ was able to bind 20 antibodies per BMP and could have potential for use in an enzyme-linked immunosorbent assay (ELISA) for selection of specific antibodies. The use of magnetic separation to isolate GPCR-displayed-BMP-antibody complexes could improve and automate the high-through-put screening process for discovery of drug targets.

Most of the applications for novel host cells have been in vaccine development and drug discovery, but the benefits of these thermo-chemically stable and non-self propagating cells can be extended into remediation and biofuels production. For

example, BMPs with surface display of metal binding proteins can be used as cost-effective biosorbents that are capable of being magnetically separated from metal-contaminated aqueous environments. Spores or BMPs displaying lignocellulases and fermentation genes will be immensely useful in achieving consolidated bioprocessing (CBP) [35] for production of biofuels. The biocatalysts will be able to endure the heat pretreatment process and be resistant to toxic fermentation by-products.

One potential concern associated with environmental release is the uncontrolled propagation of genetically modified organisms (GMO). This is especially pertinent with live vaccines and *in situ* remediation with biocatalysts. The use of novel protein display methods such as temporin and cell wall precursor UDP-MurNAc pentapeptide and novel hosts such as spores, non-living bacterial components and BMP might be a viable solution to address this risk. With the elimination of the recombinant host cells, uncontrolled-self-propagation will not occur. The cost of biocatalysts or vaccine production might increase correspondingly, but this is a small price to pay to prevent unforeseeable cost of environmental damages associated with the release of GMOs.

Concluding remarks and future perspectives

With the aid of recent advances in recombinant molecular engineering, surface-display of catalytically active enzymes or stable peptide sequences on a variety of host organisms have become routine processes. There are constant sources of new display anchor protein systems and passenger proteins. A list of environmentally relevant biocatalysts is available on the web [70]. Most of the proteins used for surface display are involved in pathogenesis (Figure 2). Currently, much of the surface protein functions

and mechanisms remain speculative. Therefore, it is important to thoroughly understand the cellular processes that involve surface proteins in bacteria, archaea and fungi. Continued investigation of the molecular mechanisms used in pathogenesis and other functions will undoubtedly result in the identification of more useful anchor proteins.

In addition to the search for novel display proteins, improvement of the existing surface-display system is needed to fully take advantage of the potential this strategy offers over intracellularly expressed proteins. Enhancement of surface expressed enzymes and anchor proteins can be attained through use of directed evolution and rational design of proteins as demonstrated in recent research [21, 58]. As our society marches towards a more technologically inclined and industrialized future, the need to find efficient methods for xenobiotic remediation and renewable energy generation has never been more pertinent. Microbial surface-display shows immense promise in improving biosorbing and biocatalytic capabilities and will undoubtedly contribute to advancements in environmental applications.

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Glossary Box

Anchor/carrier proteins are usually cell membrane associated proteins used to attach target (passenger) proteins on the cell exterior.

Biocatalysts are microbial cells expressing enzymes with catalytic activities.

C-terminal fusion is the fusion of protein to the carboxyl terminus of another protein.

***de novo* design** refers to synthesis of novel complex molecules that do not exist naturally.

DNA shuffling is a "method for *in vitro* homologous recombination of pools of selected mutant genes by random fragmentation and polymerase chain reaction reassambly" in order to artificially evolve proteins with desirable functions [71].

Enzyme-Linked ImmunoSorbent Assay (ELISA) involves immobilizing antigens on a surface and assaying for specific antibodies.

Heterologous-protein expression is the production of foreign protein by a different host organism.

Passenger protein is fused to an anchoring protein and expressed on the cell exterior.

Superfund is also known as the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA). It is a law enacted in 1980 to address cleanup, compensation and liability of abandoned hazardous waste sites in the US.

Figure Captions

Figure 1. Applications of microbial surface-display as biocatalysts (A), biosorbents (B), and biostimulants (C). Biocatalysts are used in organic pollutant degradation, biofuel production and production of enantiomerically selective organic compounds. Biosorbents displaying peptides, proteins and cyclodextrins can be used to remove metals, organic pollutants and cholesterol. Biostimulants with surface-displayed antigens or surface adhesion proteins induce antibody production, and attach to specific sites in eukaryotic cells which are used for vaccine development.

Figure 2. Natural functions of selected proteins from bacteria and yeast recently used as anchors for surface display. Six main functions are delineated from the roles of these protein anchors. Most of the proteins exhibit the functions of pathogenesis and cell-wall maintenance. According to current understanding of surface proteins, several proteins have overlapping roles. However, this role characterization is likely to change with further investigation into the molecular-basis of the mechanisms.

α–agglutinin [16, 29, 36-38, 42, 44]– mannoprotein involved in the sexual mating adhesion of *Saccharomyces cerevisiae***, AcmA** [65] – Peptidoglycan hydrolase from *Lactococcus lactis***, AIDA-I** [5, 50]- *E. coli* AIDA-I transporter protein**, CotBC** [62, 63] – Spore coat protein of *Bacillus subtilis***, Cwp2p** [54] – glycosylphosphatidylinositol-anchored yeast cell surface protein**, EstA** [48, 49, 54] - *Pseudomonas aeruginosa* EstA outer membrane esterase**, FadL** [72, 73] – outer membrane protein that binds and transports long-chain fatty acids from *E. coli***, FliCD** [15, 51]– flagellin (FliC), a major protein that makes up bacterial flagella, and the capping protein (FliD)**, Flo1p** [44, 54, 74]– flocculation functional domain from *S. cerevisiae***, HrpA** [75] – Hrp pilus structural protein from *P. syringae* **, IgA1** [5, 46] – Immunoglobulin A1 protease from *Neisseria gonorrhoeae***, INP** [13, 20, 24, 25, 27]– *P. syringae* ice nucleation protein**, Kre1p** [54, 55] – Toxin membrane receptor from *Saccharomyces cerevisiae* K1 killer strains**, Lpp-Omp** [9, 10, 14, 17] – Chimeric protein consisting of the *E. coli* lipoprotein and outer membrane protein**, Mms** [66-69] **–** Bacterial magnetic particle specific membrane protein**, OprF** [76] – *P. aeruginosa* outer membrane protein**, PgsA** [77] – A1-type transmembrane anchor from *B. subtilis***, RsaA** [53]– the component of the paracrystalline protein S-layer of *Caulobacter crescentus***, SrtA** [7, 78, 79] - Sortase A from Gram-positive bacteria**, SrtB** [7, 80] -Sortase B from Gram-positive bacteria

Table 1. Application of microbial surface-display towards xenobiotics remediation and biofuels production.

	Target	Anchor	Passenger	Host Cell	Reference
Inorganic Pollutants	Cadmium	Lpp-OmpA	Synthetic Phytochelatins	*E. coli*	[9]
	Mercury	Lpp-OmpA	Synthetic Phytochelatins	*E. coli*	[10]
	Mercury	Ice nucleation protein (INP)	MerR	*E .coli*	[13]
	Mercury	Lpp-OmpA	MerR dimers	*E. coli*	[14]
	Nickel	FliC	Dodecapeptide	*E. coli*	[15]
	Cadmium	α–agglutinin	Yeast Metallothionein (YMT)	Yeast	[16]
	Cadmium and Trichloroethylene	INP	Synthetic Phytochelatins	*Rhizobium*	[27]
Organic Pollutants	Organophosphate	Lpp-OmpA	Organophosphate Hydrolase	*E. coli*	[17]
	Organophosphate	α–agglutinin-GPI	Organophosphate Hydrolase	Yeast	[18]
	Organophosphate	INP	Organophosphate Hydrolase	*Moraxella*	[25]
	Organophosphate	INP	Carboxylesterase	*E. coli*	[20]
	Organophosphate	INP	Organophosphate Hydrolase	*E. coli*	[21]
	Organophosphate	INP	Organophosphate Hydrolase	*P. putida*	[23]
	Organophosphate	INP	Methyl Parathion Hydrolase	*P. putida JS444*	[24]
	Organophosphate	AIDA-I	Organophosphate Hydrolase	*E. coli*	[53]
Biofuels Production	Cellobiose	α–agglutinin	β-glucosidase	Yeast	[36]
	Glucan	α–agglutinin	Endoglucanase II and β–glucosidase	Yeast	[37, 38]
	Starch	α–agglutinin	Glucoamylase	Yeast	[39]
	starch	α–agglutinin	Glucoamylase and amylase	Yeast	[40, 44]
	Xylan	α–agglutinin	Xylanase II and xylosidase	Yeast	[42, 43]
	Starch	Flo1p	Amylase	Yeast	[49]

Figure 1.

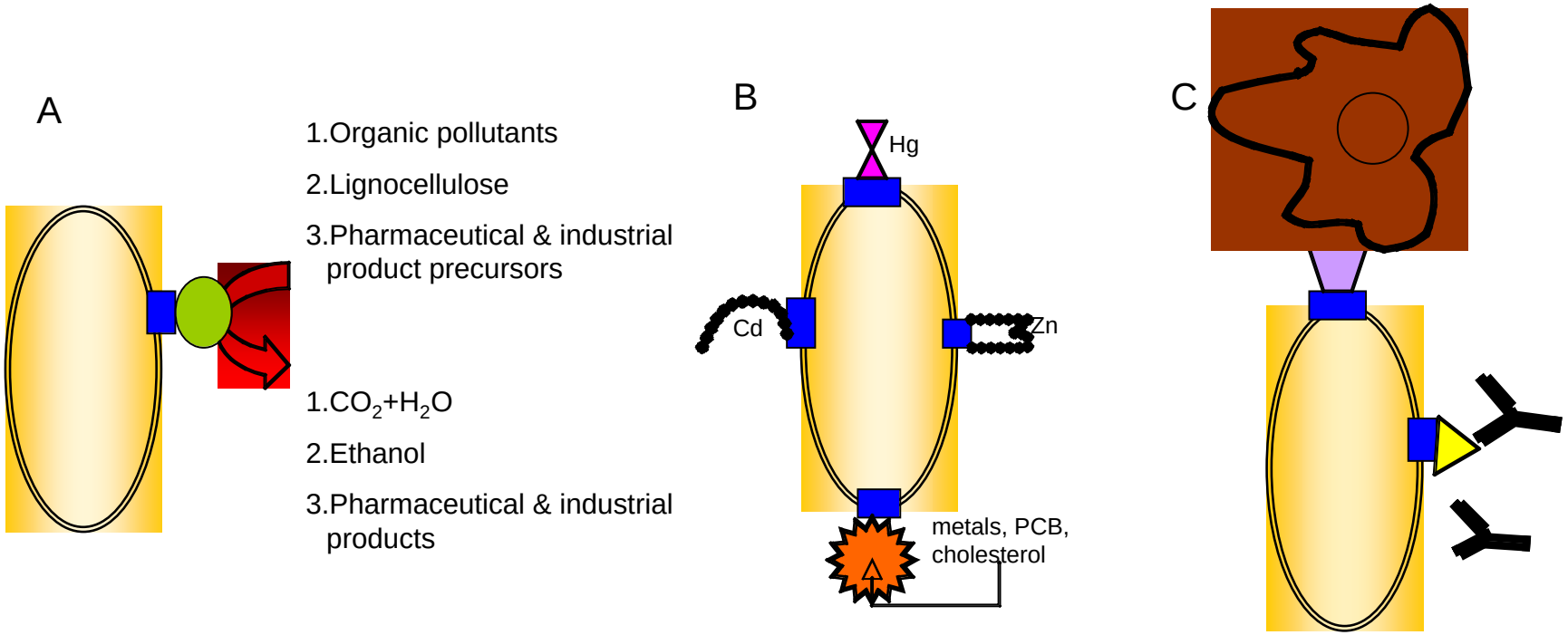
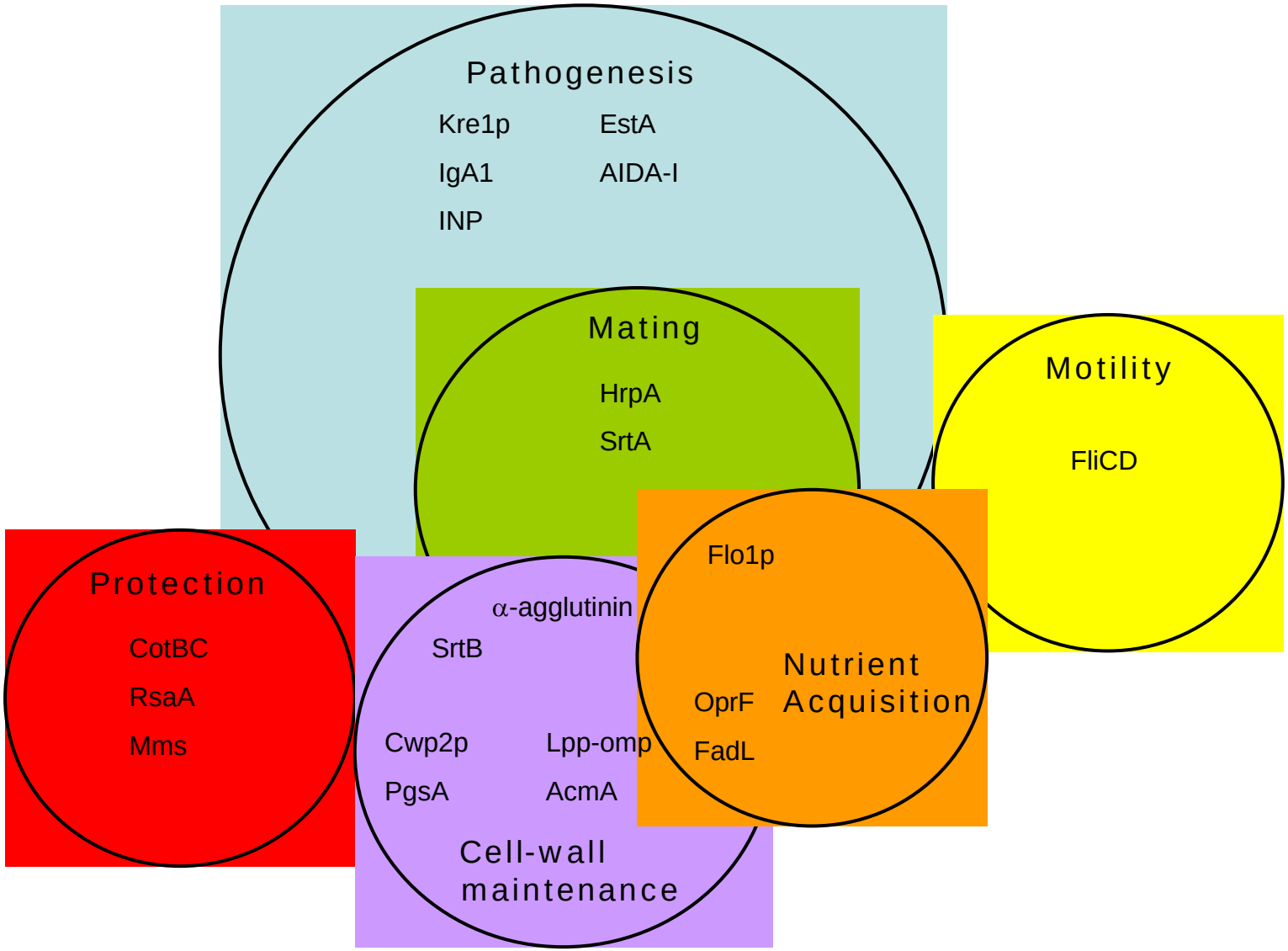

A
1.Organic pollutants
2.Lignocellulose
3.Pharmaceutical & industrial product precursors
1.CO_2+H_2O
2.Ethanol
3.Pharmaceutical & industrial products
B
Hg
Cd
Zn
metals, PCB, cholesterol
C

Figure 2.


Pathogenesis
Kre1p
EstA
IgA1
AIDA-I
INP
Mating
HrpA
SrtA
Motility
FliCD
Protection
CotBC
RsaA
Mms
α-agglutinin
SrtB
Cwp2p
Lpp-omp
PgsA
AcmA
Cell-wall maintenance
Flo1p
Nutrient Acquisition
OprF
FadL

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